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Title Page.

Title: Metabolic activity of cytochrome P450 isoforms in hepatocytes cryopreserved with wheat protein extract.

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Running Title Page.

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Abstract

The drug discovery and development process requires adequate safety testing for drug toxicity before new drugs can be administered to patients. Hepatocytes are used *in vitro* to screen compounds for hepatotoxicity, induction of drug-metabolizing enzymes such as cytochrome P450 isoforms and drug-drug interactions, and to establish human relevance for metabolism. Cryopreservation makes it possible to preserve a large quantity of functional hepatocytes. Techniques for cryopreservation of hepatocytes are mainly based on dimethylsulfoxide. However, analyses of metabolic capacities of cryopreserved hepatocytes are often limited by loss of functional integrity of hepatocytes after thawing. It is therefore necessary to improve techniques of cryopreservation. We have developed a new cryopreservation technology for mammalian cells based on a wheat protein extract (WPE). We determined whether the WPE can better preserve activities of major cytochrome P450 isoforms, both in suspension and monolayer cultures of hepatocytes. This was achieved by comparing basal and inducible or metabolic activities of isoforms CYP1A1, CYP1A2, CYP2C6, CYP2D2 and CYP3A in rat hepatocytes that were cryopreserved with WPE, relative to fresh cells and those cryopreserved with DMSO. We conclusively demonstrate that rat hepatocytes cryopreserved with WPE retain their metabolic competency and their ability to respond to classical CYP inducers, when compared to freshly isolated hepatocytes. These findings clearly show that WPE are an excellent cryopreservant for rat hepatocytes. They are an efficient, non-toxic, economic natural product and universal cryoprotectant that is superior to DMSO, which has limitations due to cellular toxicity.

The pharmaceutical industry is constantly developing new potential drug candidates for the treatment of a wide range of diseases. Adequate safety testing for drug toxicity is required by regulatory agencies (e.g. Food and Drug Administration (FDA)) before new drugs can be administered to patients. In addition to the parent drug, safety of drug metabolites must also be considered when conducting safety testing programs for new drug candidates. The liver is a major target of unacceptable human drug toxicity and is often responsible for withdrawal of drugs from clinical use, or their elimination late in the drug development process. About 20% of new drug candidates are removed from the drug development process as a consequence of unacceptable toxicity based on animal studies (Sistare and DeGeorge, 2007). Another major reason for failure of new drug candidates is adverse drug reactions (Uetrecht, 2006). In 1994, 2 million adverse drug reactions and 106,000 fatalities occurred in hospitalized patients in the US, representing an incidence of 6.7 and 0.32%, respectively (Lazarou et al., 1998).

Pharmaceutical *in vivo* testing is extremely expensive in terms of time and cost. Toxicology studies are usually conducted in animals as the basis for predicting potential human toxicities. However, there is concern about interspecies differences between animal models (e.g. rodents) and their relevance to the human context. Furthermore, current methods for assessing preclinical safety using laboratory animals often fail to detect target organ toxicity, which occurs in about 50% of cases for liver toxicity (Greaves et al., 2004). To improve the safety and efficiency of the drug discovery/development process in the future, it is important to develop improved technologies with human relevance for drug testing in preclinical trials (Sistare and DeGeorge, 2007). Detection of drug toxicity as early as possible would minimize the economic impact of withdrawing a drug late in the drug development process.

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There is considerable interest in developing new and improved, physiologically based *in vitro* models for early drug toxicity screening, which adequately reflect the human *in vivo* situation. *In vitro* systems such as organ slices and primary cell models can be used to detect a variety of target organ toxicities. For liver toxicity, hepatocytes isolated from rodents, nonrodents and humans can be useful to screen compounds *in vitro* for induction of drug-metabolizing enzymes and drug-drug interactions and to establish human relevance for metabolism (O'Brien and Siraki, 2005; Sivaraman et al., 2005). In fact, the human hepatocyte is the *in vitro* model which can produce a drug metabolic profile which is most similar to the human liver (Gomez-Lechon et al., 2003). However, the use of human hepatocytes is somewhat limited due to lack of availability and the high cost of commercially available sources. Cryopreservation offers a viable alternative to circumvent these problems.

Several methods for cryopreservation of hepatocytes have been developed and are mainly based on dimethylsulfoxide (DMSO) (Diener et al., 1993). However, analyses of metabolic capacities of cryopreserved hepatocytes are often limited by loss of functional integrity of hepatocytes after thawing. Protocols for cryopreservation of hepatocytes were optimized by the addition of sugars, osmoregulators, antioxidants or caspase inhibitors (e.g. UW solution, CryoStor Cell Freezing Media) to increase post-thaw viability and to maintain functional activity for the majority of metabolic enzymes (Li et al., 1999). However, decreased attachment efficiencies of cryopreserved hepatocytes still remain an important problem and their responsiveness in culture to inducers of CYP isoenzymes requires improvement (Li et al, 1999). Drug metabolism studies involving CYP enzyme induction require prolonged culturing of hepatocytes as attached monolayers (Li, 2007). However, inherent problems with the rapid loss of CYP activity in fresh

and cryopreserved hepatocytes still remain, when they are maintained as monolayer cultures. We recently reported the use of a plant protein extract (WPE) as a novel and non-toxic cryoprotector which improved hepatospecific functions, compared to the DMSO standard (Hamel et al., 2006).

In this report, we extend our cryopreservation studies by characterizing the activities of 4 major CYP isoforms in thawed rat hepatocytes following cryopreservation with WPE. The activities of CYP450 isoforms allow a critical evaluation of the quality and metabolic capacity of cryopreserved cells upon thawing. We have determined whether the WPE can better preserve activities of CYP isoforms, both in suspension and monolayer cultures of hepatocytes. This was achieved by comparing basal and inducible or metabolic activities of isoforms CYP1A1, CYP1A2, CYP2C6, CYP2D2 and CYP3A in rat hepatocytes that were cryopreserved with WPE, relative to fresh cells and those cryopreserved with DMSO.

Methods

Chemicals

Collagenase, insulin, Williams' medium E (WME), DMSO, dexamethasone, phenobarbital, dextromethorphan, propidium iodide (PI) and other chemicals were from Sigma Chemical Company (St. Louis, MO). Leibovitz medium (L-15), gentamicin, MEM vitamins and Matrigel were from Gibco/Life Technologies (Burlington, ON). Fetal bovine serum (FBS) was from Medicorp (Montreal, QC). 3-Cyano-7-ethoxycoumarin (CEC), 7-methoxy-4-(trifluoro-methyl)coumarin (MFC) and 3-[2-(N,N-diethyl-N-methylammonium) ethyl]-7-methoxy-4-methylcoumarin (AMMC) were purchased from BD Gentest (Mississauga, ON) and [3-[(3,4-difluorobenzyl)oxy]-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]furan-2(5H)-one] (DFB) was offered generously by Merck Frosst (Montreal, QC) (Nicoll-Griffith et al., 2004).

Plant Materials and Growth Conditions

Winter wheat genotype (*Triticum aestivum* L. cv Clair, LT_{50} (lethal temperature that kills 50% of seedlings) -19^{o}C) was used in this study. Wheat plants were grown and treated as previously described (Danyluk et al., 1998). Briefly, plants were grown for 10 days under a 16 hour/day photoperiod with a light intensity of 250µmol/m^{2}s at 20^{o}C.

Total Protein Extraction

The aerial parts of seedlings were collected and blended until a homogeneous solution was obtained with cold ultrapure water. The homogenate was filtered through three layers of miracloth and centrifuged at 30,000g for 45 min at 4^{o}C. The pH of the supernatant was adjusted to 7.4 and sterilized using a 0.22 mm filter. The extract was concentrated by freeze drying and

stored at -20^{o}C. The dried powder extract was resuspended in ice-cold WME medium before being added to the hepatocyte suspension (Hamel et al., 2006).

Hepatocyte Isolation

Hepatocytes were isolated from male Sprague–Dawley rats (140–180g) (Charles River Canada, Saint-Constant, QC), by the two-step collagenase digestion technique (Seglen, 1976; Guillemette et al., 1993). Animals were maintained and handled in accordance with the Canadian Council on Animal Care guidelines (Olfert et al., 1993) for care and use of experimental animals. Cell viability was evaluated by flow cytometry (FACScan, Becton Dickinson, Oakville, ON) with 2 µM PI (Reader et al., 1993).

Cryopreservation of Hepatocytes

Immediately after isolation, the hepatocyte suspension (5 x 10^6 cells/mL) was added to ice-cold WME medium supplemented with 10% FBS and 60 mg of WPE in cold cryovials. Positive (15% DMSO and 50% FBS) and negative (WME) controls were also prepared. Cells in cryovials were frozen at a cooling rate of 1^0C/min in a controlled freezing container (Nalgene, Rochester, NY) to -80^0C for 1 day, and then transferred to liquid nitrogen for 7 days (Hamel et al., 2006).

Thawing of Cryopreserved Hepatocytes

Frozen cells were thawed quickly by gentle agitation in a 37^0C water-bath and viability was determined with 2 µM PI (70-80%). Immediately after thawing, the hepatocyte suspension was diluted 10-fold with cold WME medium. After centrifugation (4^0C, 50xg, 2 min), hepatocytes were suspended in 10 mL of WME medium and washed twice. After washing, dead cells were

removed by 30% isotonic Percoll centrifugation. Recovery of cells after this step was 85%. The viability of rat hepatocytes following cryopreservation with WPE was significantly higher ($P < 0.05$) at 72.15 ± 1.35%, compared to 66.93 ± 2.25% for DMSO. For fresh cells, viability was 92.67 ± 2.89%. Post-thaw viability was very low in negative (WME) controls (1.6%).

For tests in suspension, hepatocytes were adjusted to $1x10^3$ cells/µL. For monolayer cultures, hepatocytes ($2.5x10^5$ cells/mL) were plated in 4-well Corning plastic tissue culture plates, noncoated or coated with Matrigel, in WME medium supplemented with insulin (0.2 mg/mL) and gentamicin (50 mg/mL) in a humidified atmosphere of 5% CO_2 and 95% air at 37^0C. After 3 h, medium was changed and cells were incubated overnight in L-15 medium supplemented with insulin and gentamicin.

Cellular Morphology

Morphology of hepatocytes, in regular plastic tissue culture plates or in those coated with Matrigel, was evaluated by microscopy (Carl Zeiss Ltd., Montreal, QC). Photographs were taken by digital camera (Camera CCD, Sony DXC-950P; Empix Imaging, Inc., Mississauga, ON) and images were analyzed using Northern Eclipse software.

Viability

Lactate dehydrogenase (LDH) activity was determined in the medium of seeded, adherent hepatocytes (Moldeus et al., 1978). The hepatocyte culture medium was removed and activity of LDH released into the medium from adherent cells was quantified. The total amount of LDH activity was obtained by lysis of live cells with 10% Triton X-100 (Moffatt et al., 1996).

Attachment efficiency was evaluated by LDH activity in freshly isolated and attached cryopreserved hepatocytes after 24 h in culture.

Enzymatic Activity of CYP Isoforms

The basal enzymatic activities were measured in hepatocyte suspensions immediately post-thaw. For induction of CYP activities, hepatocytes were cultured for 24 h and then treated for 48 h with benzo-[a]-pyrene (10 µM, CYP 1A1), phenobarbital (50 µM, CYP 2C6), or dexamethasone (50 µM, CYP 3A1/2). Dextromethorphan (50 µM) was used as a metabolic substrate for CYP 2D2. Cells were washed twice with PBS to remove these compounds. CYP activities were measured using fluorescent substrates for CYP450 isoforms 1A1/2 (CEC, ex: 410nm, em: 450nm), 2D2 (AMMC, ex: 410nm, em: 450nm), 2C6 (MFC, ex: 410nm, em: 500nm) and 3A1/2 (DFB, ex: 340nm, em: 450nm). Cells were incubated with 25 µM of different substrates for different times between 0 and 30 min. After the incubation, 150 µL of cell suspension was added to 150µL of acetonitrile, or 60% acetonitrile with 40% Tris base (pH 10, DFB). Fluorescence was detected using a spectrofluorimeter (SPECTRAFluor Plus, Tecan, CA).

Statistical Analysis

Results were expressed as mean ± SEM of at least three replicate dishes for each condition with a minimum of three experimental repeats using different cell preparations from separate animals. Comparison between groups and analysis for differences between means of control and treated groups were performed using ANOVA followed by the post hoc test Newman–Keuls ($P<0.05$). The threshold for statistical significance was: $P<0.05$ (*), $P<0.01$ (**), and $P<0.001$ (***).

Results

Cellular Morphology of Rat Hepatocytes Cryopreserved with WPE

It is widely recognized that cellular adherence is disturbed in cryopreserved cells upon thawing (Terry et al., 2006a). Consequently, we evaluated whether cryopreservation with WPE could improve the cellular morphology of hepatocytes following thawing. Freshly isolated hepatocytes exhibited pronounced differences in morphology, depending on whether they were cultured on regular plastic dishes or on Matrigel-coated dishes (Fig. 1). Fresh cells exhibited a spread-out phenotype on the plastic surface, when compared to cryopreserved cells. However, when cultured on Matrigel, fresh cells rolled together and formed three dimensional spheroids. When hepatocytes were cryopreserved with WPE or DMSO, they were still much rounder than fresh cells. The cryopreserved cells did not form spheroids on Matrigel, in contrast to freshly isolated cells. However, cells that were cryopreserved with WPE did appear to form small colonies of cells, more so than cells that were cryopreserved with DMSO. Cryopreservation with WPE improved the morphology of hepatocytes to some extent compared to those cryopreserved with DMSO. However, the rounded morphology was still quite different from that of fresh cells. Attachment efficiencies for cells that were cryopreserved with WPE and DMSO were similar with values of 50.9 ± 2.9% and 50.3 ± 4.1%, respectively, relative to fresh hepatocytes, as reference control (100%).

Basal Activities of Cytochrome P450 Isoforms in Cryopreserved Hepatocytes

The activities of CYP450 isoforms are one of the most unstable functions in isolated hepatocytes (Grant et al., 1985; Paine, 1990) and are highly sensitive to changes in cellular environment. For example, activities of P450 isoforms such as CYP1A2 and CYP3A4 decrease by approximately

50% per day in culture (Li, 2007). We therefore determined whether the activities of 4 major CYP isoforms would remain at levels comparable to fresh cells, in suspension and monolayer cultures of rat hepatocytes that were cryopreserved with WPE.

The basal activities of CYP3A1/2 and CYP2D2 were similar in both WPE-cryopreserved and freshly isolated hepatocytes (Fig. 2A, B). However, for hepatocytes that were cryopreserved with DMSO, the enzymatic activities of isoforms CYP3A1/2 and CYP2D2 were decreased by at least 50% (Fig. 2A, B), when compared to fresh hepatocytes. For CYP1A1/2 and CYP2C6, basal activities in hepatocytes that had been cryopreserved with WPE or DMSO were similar and slightly higher than those in fresh cells (Fig. 2C, D). These data show that cryopreservation of hepatocytes with WPE is advantageous when compared to DMSO.

Induction of Cytochrome P450 Isoforms

Fresh and thawed cryopreserved hepatocytes were maintained in culture during 3 days, either in plastic or Matrigel-coated dishes. Subsequently, the induction or metabolic activity of the 4 CYP isoforms was determined in hepatocytes that had been cryopreserved with WPE, compared to fresh cells and those cryopreserved with DMSO.

A. Regular Plastic Dishes

For isoforms CYP3A1/2, basal activity after 3 days in culture was lower for cryopreserved cells, when compared to fresh hepatocytes (Fig. 3A). After induction with dexamethasone, CYP3A1/2 activity was also lower for cryopreserved cells, compared to fresh cells (Fig. 3A). However, induced CYP3A1/2 activity was higher in hepatocytes that were cryopreserved with WPE,

compared to DMSO. The induction of enzymatic activity (induced/non-induced: (I/NI)) for fresh cells (2.08, 30 min) and those cryopreserved with WPE (2.02) was similar (Fig. 3B), whereas it was lower for cells cryopreserved with DMSO (1.69).

The basal activity of isoform CYP2D2 after 3 days in culture was higher for hepatocytes that were cryopreserved with DMSO, than for fresh cells and those cryopreserved with WPE (Fig. 4A). CYP2D2 activity was increased by dextromethorphan in fresh and WPE-cryopreserved cells (Fig. 4A), whereas it decreased in cells that were cryopreserved with DMSO. The increase in enzymatic activity for fresh and WPE-cryopreserved cells was similar (4.57, 15 min) (Fig. 4B), whereas that for cells cryopreserved with DMSO was much lower (0.41, 15 min).

For the isoforms CYP1A1/2, the basal activities after 3 days in culture were similar for cryopreserved cells, but lower than in fresh hepatocytes (Fig. 5A). After induction with benzo[a]pyrene, the levels of CYP1A1/2 activity were higher for hepatocytes that were cryopreserved with WPE, compared to DMSO, but lower than in fresh cells. The induced enzymatic activities for cells that were cryopreserved with DMSO (24.31, 15 min) and WPE (22.29) were higher than for fresh hepatocytes (17.68) (Fig. 5B).

For hepatocytes that were cryopreserved with WPE, basal activity for the isoform CYP2C6 after 3 days in culture was similar to fresh hepatocytes after 15 min, but slightly lower after 30 min (Fig. 6A). However, activity was lower for cells cryopreserved with DMSO, when compared to WPE. After induction with phenobarbital, CYP2C6 activity showed the same trend as for basal activity (Fig. 6B). The induced enzymatic activities for fresh (4.56, 15 min) and WPE-cryopreserved hepatocytes (4.41, 15 min) were similar, whereas that for DMSO-cryopreserved cells was lower (3.39) (Fig. 6B).

In general, the rates of induction of activities for the CYP isoforms were similar when fresh hepatocytes and those cryopreserved with WPE were cultured for 3 days in regular plastic dishes. Apart from CYP1A1/2, they were consistently lower in cells that had been cryopreserved with DMSO.

B. Matrigel-coated Dishes

There was a general increase in activities of the 4 cytochrome P450 isoforms for fresh and cryopreserved cells when cultured on Matrigel-coated dishes (Figs. 3-6, panels C,D), compared to regular plastic dishes (Figs. 3-6, panels A,B).

For the CYP3A1/2 isoforms, the basal activities and rates of induction of activity, after 3 days of culture on Matrigel-coated dishes (Fig. 3C, D), were qualitatively similar to those observed for hepatocytes that were cultured in regular plastic dishes (Fig. 3A, B). The only difference was that activities and induced activities were approx. 2-fold higher for hepatocytes that had been cultured on Matrigel. The induced enzymatic activities for cells that were cryopreserved with WPE (4.21, 5 min) were again similar to fresh hepatocytes (4.54) and higher than for those cryopreserved with DMSO (3.3) (Fig. 3D).

The basal activities of the isoform CYP2D2 after 3 days of culture on Matrigel matrix were similar for cryopreserved and fresh hepatocytes (Fig. 4C). When cells were treated with dextromethorphan, metabolic activities for CYP2D2 were also similar after 15 min, but after 30 min, activity in fresh cells was higher than for the other cell types (Fig. 4C). The increases in enzymatic activities were similar for fresh and WPE-cryopreserved cells (4.85 and 5.10, 15 min),

but higher than for those cryopreserved with DMSO (4.19) (Fig. 4D). However, in contrast to culture on plastic dishes, the activity of CYP2D2 was restored when DMSO-cryopreserved hepatocytes were cultured on Matrigel surfaces (Fig. 4B, D).

The basal activities of isoforms CYP1A1/2 were similar for cryopreserved and fresh hepatocytes, after 3 days of culture on the Matrigel matrix (Fig. 5C). After induction with benzo[a]pyrene, CYP1A1/2 activity was lower in cells that were cryopreserved with WPE, when compared to fresh hepatocytes, but still higher than for those cryopreserved with DMSO (Fig. 5C). The induced enzymatic activity for cells cryopreserved with WPE (42.43, 15 min) was similar to that of fresh hepatocytes (39.21), but again higher than for those cryopreserved with DMSO (31.76) (Fig. 5D).

For isoform CYP2C6, the basal and phenobarbital-induced activities, after 3 days of culture on Matrigel-coated dishes (Fig. 6C), were qualitatively similar to those observed for hepatocytes that were cultured in uncoated dishes (Fig. 6A). The induced enzymatic activities for fresh (5.29) and cryopreserved hepatocytes (WPE, 4.93; DMSO, 5.28) were similar (Fig. 6D).

These results show that the culture of fresh and cryopreserved hepatocytes on Matrigel-coated dishes improves the basal and induced or metabolic activities of the 4 CYP isoforms. There was a notable improvement in activity of CYP2D2 for DMSO-cryopreserved cells that were cultured on Matrigel matrix, compared to uncoated surfaces. Again, when hepatocytes were cultured for 3 days in Matrigel-coated dishes, the induced or metabolic activities of the 4 CYP isoforms in fresh and WPE-cryopreserved hepatocytes were similar, whereas they were generally lower in cells cryopreserved with DMSO. Together, these findings demonstrate that cryopreservation of

hepatocytes with WPE is clearly advantageous and comparable to fresh hepatocytes, in terms of preserving activities of the 4 major CYP isoforms, when compared to the DMSO standard.

Discussion

We previously reported that WPE is an efficient cryoprotective agent for primary cultures of rat hepatocytes (Hamel et al., 2006). Cryopreservation of hepatocytes with WPE retained adequate viability, attachment efficiencies, hepatospecific functions such as albumin secretion and biotransformation of ammonium to urea, and capacity to respond to inducers of CYP1A1 and CYP2B (Hamel et al., 2006). The significant new findings in this study are that, upon thawing, the activities of 4 major drug-metabolizing enzymes of the cytochrome P450 family are similar in rat hepatocytes that were cryopreserved with WPE and freshly isolated cells. When cells cryopreserved with WPE were cultured as attached monolayers for 3 days upon thawing, metabolic activities of the CYP isoforms 3A1/2, 2D2 and 2C6 in response to classical CYP inducers were equivalent to those in fresh hepatocytes, but slightly higher for CYP1A1/2. Basal enzymatic activities of the 4 CYP isoforms were similar in suspensions of fresh and WPE-cryopreserved hepatocytes.

The drug discovery process utilizes hepatocytes in suspension and in monolayer cultures. Due to their limited lifespan (approx. 5h), suspension cultures of hepatocytes can be used for applications in the early drug discovery process such as hepatic metabolic stability, P450 inhibition and drug transporter activities (Li, 2007). However, P450 induction studies remain the 'gold standard' for detecting drug-drug interactions, which are required by the FDA. The evaluation of P450 induction requires the use of monolayer cultures of hepatocytes («plateable» hepatocytes) that can be maintained in culture for 2 to 3 days. Cryopreservation with WPE could be a valuable tool in drug discovery since hepatocytes retained activity of the 4 CYP isoforms both in suspension and in monolayer culture.

Our findings show that the activities of the 4 CYP isoforms were markedly increased when cryopreserved and fresh hepatocytes were cultured on dishes pretreated with an attachment substrate such as Matrigel. The Matrigel matrix is a basement membrane preparation which contains extracellular matrix proteins such as laminin, collagen IV and entactin. It provides a physiologically relevant environment which better reflects the *in vivo* context. For culture on Matrigel, activities of the 4 CYP isoforms were similar in fresh cells and those cryopreserved with WPE, whereas they were lower in cells cryopreserved with DMSO, with the exception of CYP2C6.

There were marked differences in morphology when hepatocytes were cultured on Matrigel matrix versus plastic surfaces. Differences were more pronounced for fresh than cryopreserved hepatocytes. However, hepatocytes that were cryopreserved with WPE or DMSO maintained a rounded morphology on both plastic and Matrigel surfaces. An important point is that the rounded morphology did not affect responses of cells that had been cryopreserved with WPE to classical CYP inducers.

The attachment efficiencies of hepatocytes that had been cryopreserved with WPE or DMSO were 50% compared to freshly isolated hepatocytes, when cultured as monolayers on both plastic and Matrigel-coated surfaces. One of the major limitations of hepatocyte cryopreservation is that low attachment efficiencies are obtained upon thawing (Li et al., 1999; Li, 2007). Attachment efficiencies for DMSO-cryopreserved hepatocytes from various animal species are generally in the range of 50%, compared to fresh cells (Chesne and Guillouzo, 1988; Chesne et al., 1993).

Attachment efficiencies are even lower (5-40%) for «plateable» cryopreserved human hepatocytes (Zvibel et al., 2002; Terry et al., 2006c). This is a major problem since cell adhesion and cell-cell contacts have an impact on metabolic activities and functional integrity of hepatocytes. Despite the low attachment efficiencies, cryopreserved rat hepatocytes can still be cultured as reliably as freshly isolated hepatocytes. Several studies have shown that rat, mouse and human hepatocytes can retain adequate viability and metabolic competency following cryopreservation with DMSO (de Sousa et al., 1996; Guillouzo et al., 1999).

Our findings show that the induced or metabolic activities of CYP450 isoforms in cells that were cryopreserved with WPE were consistently similar to those observed in freshly isolated hepatocytes. However, induction of CYP activity in cells cryopreserved with DMSO was equivalent or slightly lower, compared to WPE or fresh cells. Our findings suggest that WPE is a more suitable cryopreservant for primary hepatocyte cultures than DMSO.

Activities of the phase II enzymes, UDP-glucuronosyl S-transferases (UGT) and sulphotransferases, were well preserved in rat hepatocytes that were cryopreserved with DMSO (Hewitt and Utesch, 2004). On the other hand, glutathione S-transferase and glutathione reductase activities of cryopreserved human hepatocytes were reduced to less than 40% of the enzyme activity of fresh hepatocytes (Coundouris et al., 1993). Drug transporter activities were also well preserved in cryopreserved rat hepatocytes (Houle et al., 2003). Cytochrome P450 enzymes such as CYP3A, CYP2C and CYP2D were generally well preserved in hepatocytes following cryopreserved with DMSO (Shaddock et al., 1993; de Sousa et al., 1996; Hengstler et al., 2000a; Hengstler et al., 2000b; Houle et al., 2003; Hewitt and Utesch, 2004). However,

CYP2C activity (tolbutamide hydroxylase activity) in suspensions of cells that were cryopreserved with DMSO was lower, compared to fresh cells (Hewitt and Utesch, 2004). It was reported that low temperatures can induce conformational changes in the structure of CYP450 (Nardid et al., 1997).

Cryopreserved hepatocytes are often less responsive to enzyme-inducing agents than fresh hepatocytes (Terry et al., 2006a). Rifampicin induced testosterone metabolism by 4.5-fold in fresh hepatocytes, but only 2-fold in cryopreserved cells (Reinach et al., 1999). Previous studies reported that hepatocytes were responsive to CYP1A and CYP3A inducers following cryopreservation with DMSO (Reinach et al., 1999; Silva et al., 1999). However, the specific activity, after induction, was lower than in freshly isolated cells.

Classical agents such as DMSO are used to protect cells from dehydration caused by the formation of intracellular ice during cryopreservation and minimise potential damage due to osmotic imbalance. However, DMSO causes membrane damage and is toxic to hepatocytes, particularly at temperatures such as 25^{o}C and 37^{o}C, and must be removed rapidly from contact with cells during post-thaw processing (Terry et al., 2006b). DMSO causes structural and metabolic impairment of hepatocytes and compromises their ability to respond to enzyme-inducing agents and hormones (Diener et al., 1993). Therefore, hepatocytes cryopreserved with DMSO do not display the metabolic state of fresh cells, and their use can lead to erroneous interpretation of results. It is therefore important to develop non-toxic alternatives to replace DMSO as a cryopreservation agent, particularly for sensitive cell types such as hepatocytes.

We previously demonstrated that cryoprotective activity appears to be specific to proteins in wheat extracts, since other proteins such as bovine serum albumin (BSA), E. coli proteins, or fetal bovine serum (FBS) did not show any cryoprotective activity in hepatocytes (Hamel et al., 2006). However, the cryoprotective activity of proteins in extracts from other types of plants cannot be ruled out.

The higher cryopreservation capacity of WPE compared to DMSO is probably explained by protective effects of WPE against cryopreservation-induced degradation of membrane proteins by unknown processes. Overwintering plants such as the hardy winter wheat genotype (*Triticum aestivum* L. cv Clair) accumulate protective compounds that ensure survival of the plant during freezing. These protective compounds include sugars (glucose, fructose, sucrose, trehalose), antioxidants, amino acids and proteins such as antifreeze proteins and dehydrins. These proteins have the potential to protect cells against dehydration caused by freezing. The wheat proteins may agglutinate at the plasma membrane, thus decreasing the aqueous flow whose regulation is very important in order to minimize degradation and avoid the loss of viability. These proteins may also protect cells against damage by stabilizing proteins and membranes during freezing.

This study conclusively demonstrates that rat hepatocytes cryopreserved with WPE retain their metabolic competency and their ability to respond to classical CYP inducers upon thawing, when compared to freshly isolated hepatocytes. Viability and CYP metabolic studies were also carried out using hepatocytes from different rats that were cryopreserved with different lots of WPEs for longer periods of time, ranging from to several months up to a maximum of 4 years. Similar data were obtained whether hepatocytes were cryopreserved for 7 days, several months or 4 years

(data not shown). These findings clearly show that WPE is an excellent cryopreservant for long-term storage of primary hepatocytes. They are an efficient, non-toxic, economic natural product and universal cryoprotectant that is superior to DMSO, which has limitations due to cellular toxicity. This technology is highly relevant to the drug discovery process and is currently being validated in human hepatocytes. During recent years, hepatocytes isolated from unused donor livers are being used for clinical transplantation in patients with acute liver failure and liver-based metabolic defects. Development and optimization of cryopreservation protocols for human hepatocytes will provide a solution to increase the availability of hepatocytes with good thawed function for their use in transplantation (Terry et al., 2006a).

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Footnotes

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Legends to Figures

Figure 1: Cellular morphology of rat hepatocytes cryopreserved with WPE

Cellular morphology was visualized 24 h after seeding thawed rat hepatocytes on either regular plastic dishes or Matrigel-coated dishes, following cryopreservation for 7 days in WME 10% FBS supplemented with 15% DMSO and 50% FBS (DMSO) or with 60 mg of WPE (WPE). Freshly isolated hepatocytes (FRESH) served as reference. Hepatocytes ($250x10^3$) were visualized by microscopy (Magnification 100X). Photographs are from one representative experiment, which was repeated at least three times.

Figure 2: Basal activity of 4 cytochrome P450 isoforms in rat hepatocytes cryopreserved with WPE: comparison with DMSO-cryopreserved and fresh cells

Enzymatic activity of 4 cytochrome P450 isoforms was evaluated using fluorescent probes in cell suspensions immediately post-thaw. A) CYP3A1/2: DFB B) CYP2D2: AMMC, C) CYP1A1/2: CEC and D) CYP2C6: MFC. Freshly isolated hepatocytes (Fresh) served as reference. Data (mean ± SEM) represent duplicate measurements from four experiments with different cell preparations (n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3: Effect of cryopreservation of rat hepatocytes with WPE on metabolic activity of cytochrome P450 isoform CYP3A1/2

Rat hepatocytes were cryopreserved for 7 days in WME supplemented with 15 % DMSO and 50 % FBS (DMSO) or 60 mg of WPE. Hepatocytes were thawed and seeded in culture for 24 h. Dexamethasone was then used to induce CYP activity during 48h. Enzymatic activity of CYP3A1/2 was evaluated using DFB for hepatocytes in culture on regular plastic dishes and

expressed as A) relative fluorescence and B) induced/non-induced activity. C) Relative fluorescence and D) induced/non-induced (I/NI) activity are shown for cells in culture on Matrigel-coated dishes. Freshly isolated hepatocytes (Fresh) were cultured for 72h and served as reference. Data (mean ± SEM) represent duplicate measurements from four experiments with different cell preparations (n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NI= non-induced and I=induced.

Figure 4: Metabolic activity of cytochrome P450 isoform CYP2D2 in hepatocytes cryopreserved with WPE

Rat hepatocytes were cryopreserved for 7 days in WME supplemented with 15 % DMSO and 50 % FBS (DMSO) or 60 mg of WPE. Hepatocytes were thawed and seeded in culture for 24 h. Dextromethorphan (Dextro) was then used to increase metabolic activity during 48h. Enzymatic activity of CYP2D2 was evaluated using AMMC for hepatocytes in culture on regular plastic dishes and expressed as A) relative fluorescence and B) enzymatic activity (dextro/no dextro). C) Relative fluorescence and D) enzymatic activity (dextro/no dextro) are shown for cells in culture on Matrigel-coated dishes. Freshly isolated hepatocytes (Fresh) were cultured for 72h and served as reference. Data (mean ± SEM) represent duplicate measurements from four experiments with different cell preparations (n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NI= non-induced and I=induced.

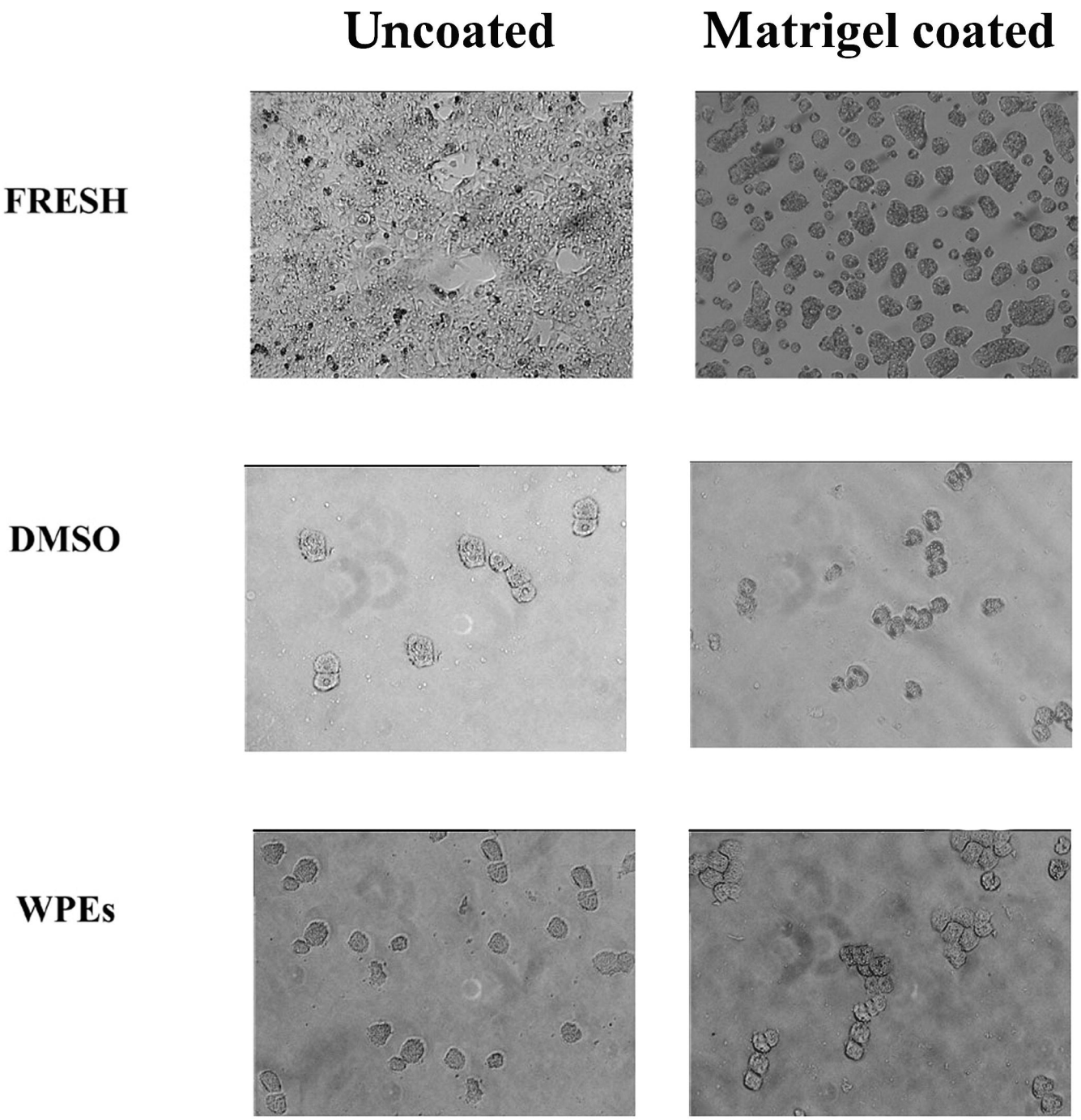
Figure 5: Metabolic activity of cytochrome P450 isoform CYP1A1/2 in cryopreserved rat hepatocytes: effects of WPE

Rat hepatocytes were cryopreserved for 7 days in WME supplemented with 15% DMSO and 50 % FBS (DMSO) or 60 mg of WPE. Hepatocytes were thawed and seeded in culture for 24 h. Benzo-[a]-pyrene was then used to induce CYP activity during 48h. Enzymatic activity of CYP1A1/2 was evaluated using CEC for hepatocytes in culture on regular plastic dishes and expressed as A) relative fluorescence and B) induced/non-induced activity. C) Relative fluorescence and D) induced/non-induced activity are shown for cells in culture on Matrigel-coated dishes. Freshly isolated hepatocytes (Fresh) hepatocytes were cultured for 72h and served as reference. Data (mean ± SEM) represent duplicate measurements from four experiments with different cell preparations (n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NI= non-induced and I=induced.

Figure 6: Effects of cryopreservation with WPE on metabolic activity of cytochrome P450 isoform CYP2C6

Rat hepatocytes were cryopreserved for 7 days in WME supplemented with 15 % DMSO and 50 % FBS (DMSO) or 60 mg of WPE. Hepatocytes were thawed and seeded in culture for 24 h. Phenobarbital was then used to induce CYP activity during 48h. Enzymatic activity of CYP2C6 was evaluated using MFC for hepatocytes in culture on regular plastic dishes and expressed as A) relative fluorescence and B) induced/non-induced activity. C) Relative fluorescence and D) induced/non-induced activity are shown for cells in culture on Matrigel-coated dishes. Freshly isolated hepatocytes (Fresh) were cultured for 72h and served as reference. Data (mean ± SEM) represent duplicate measurements from four experiments with different cell preparations (n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$. NI= non-induced and I=induced.

Figure 1



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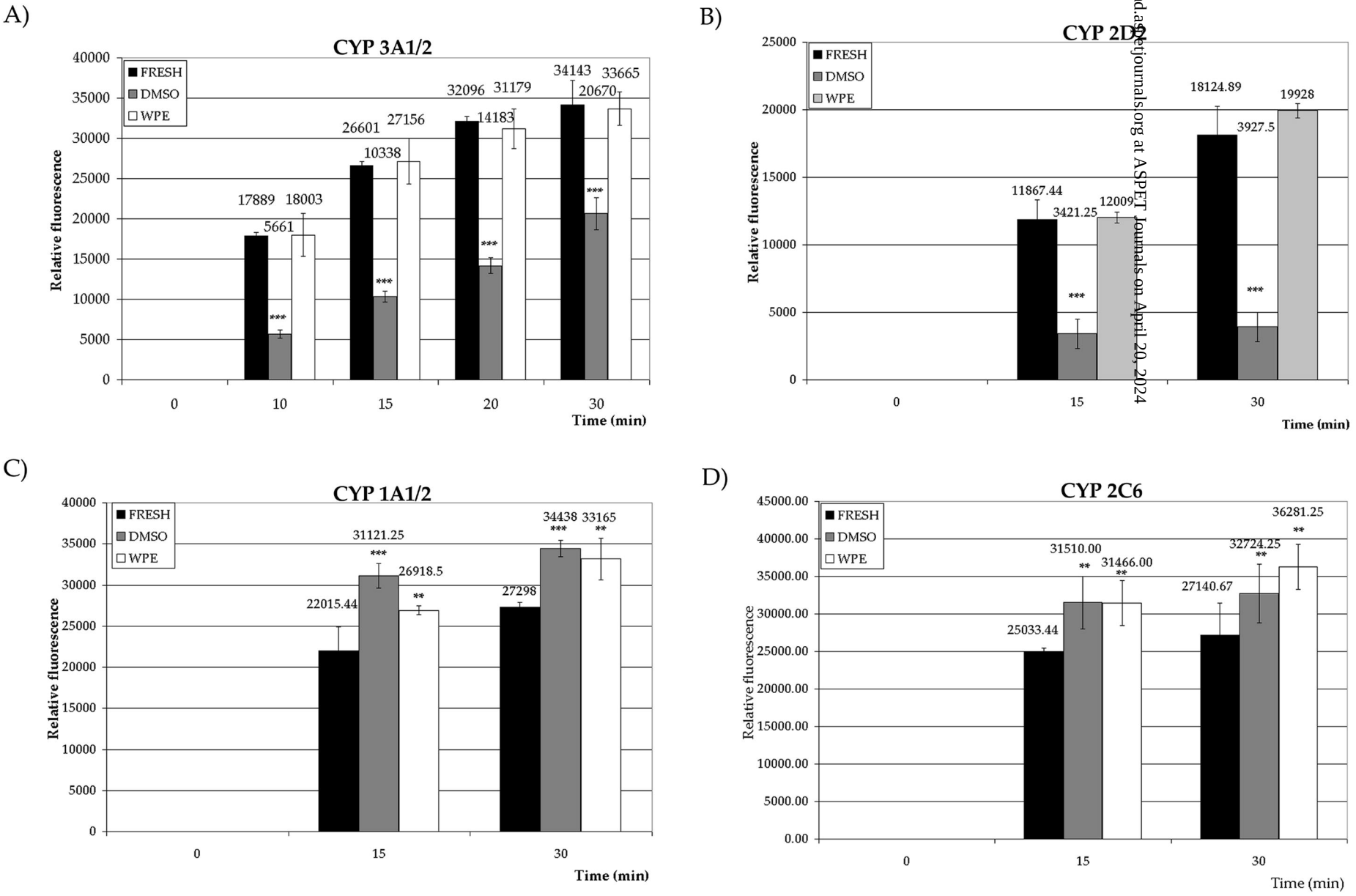

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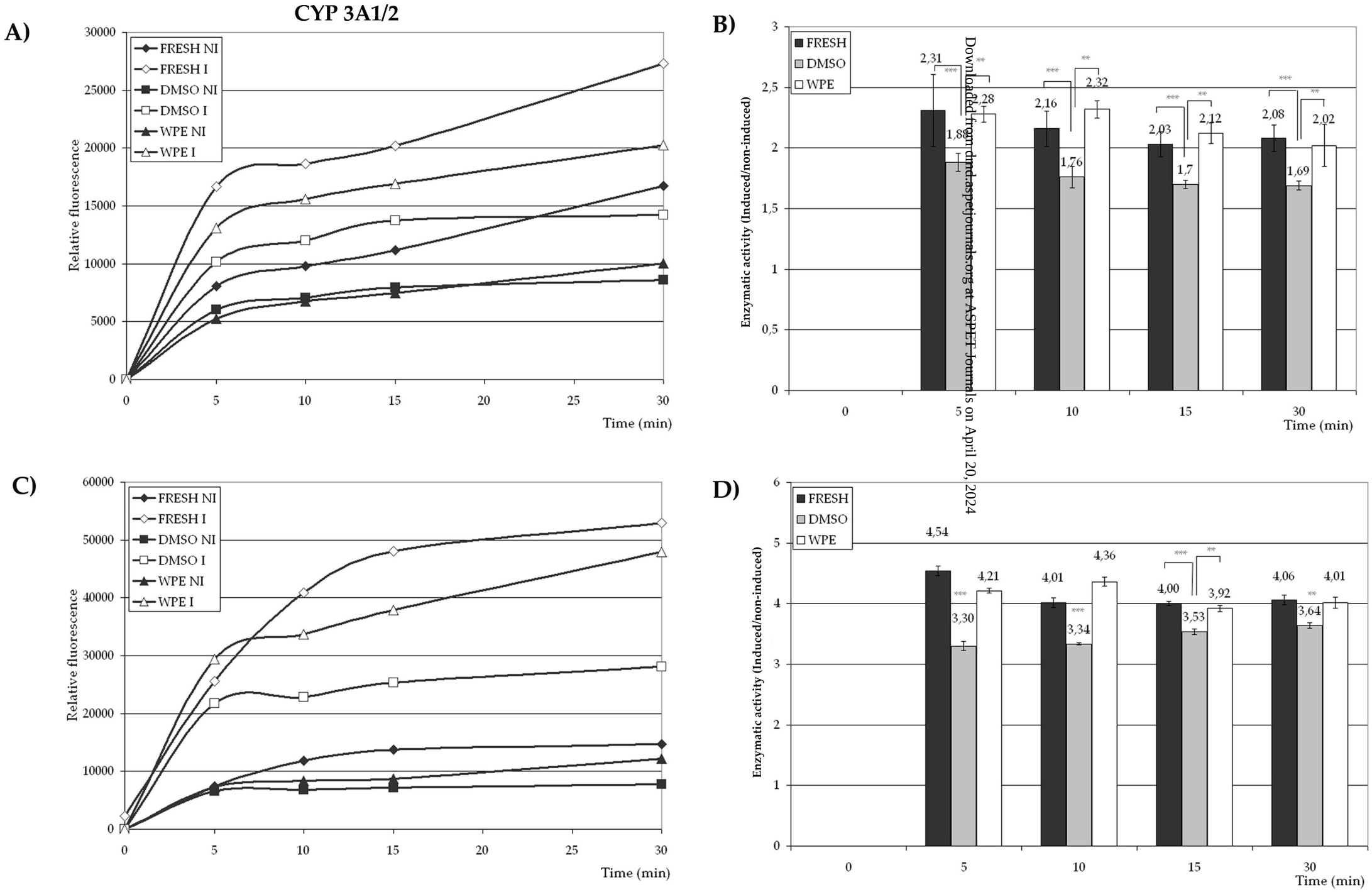

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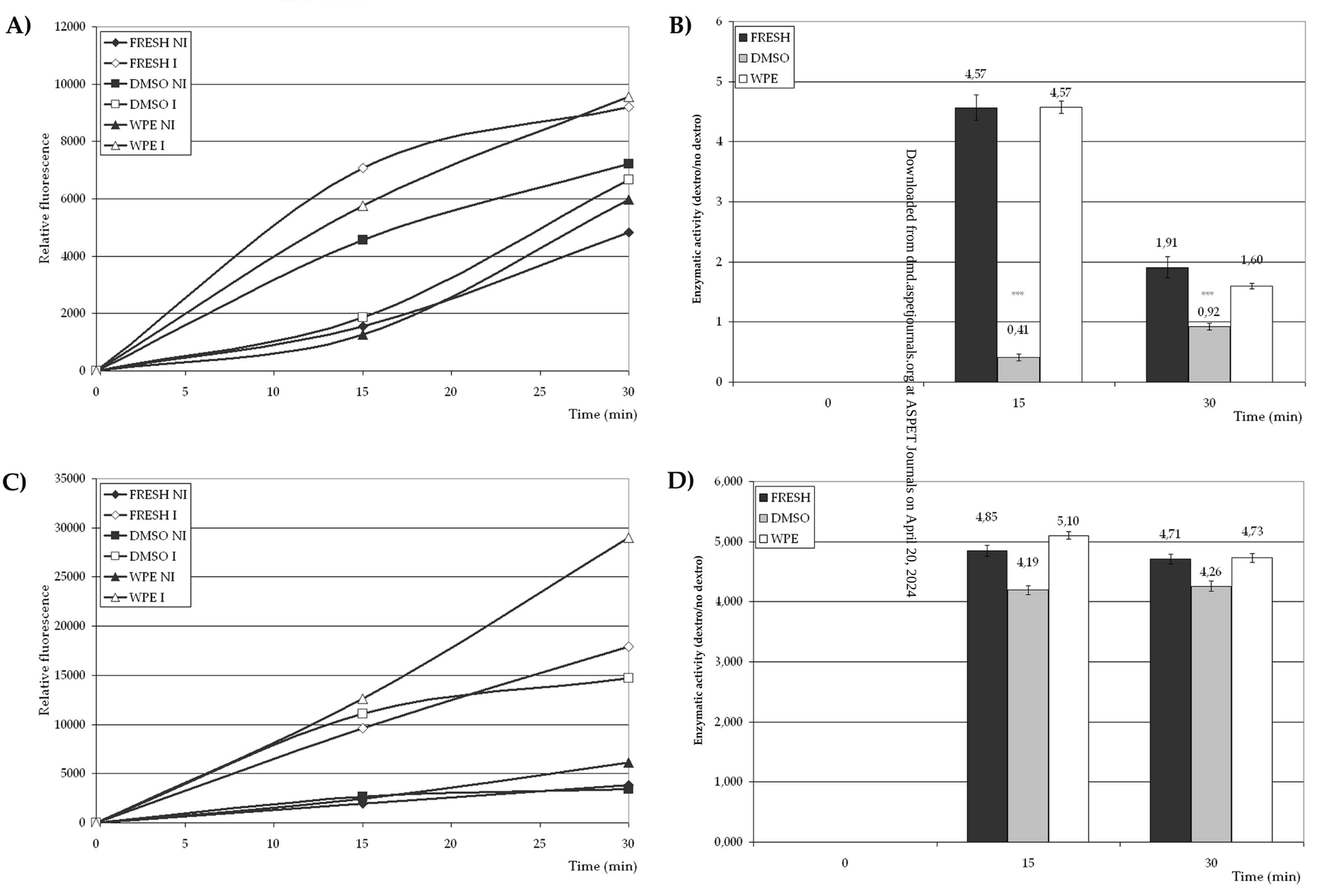

Figure 5

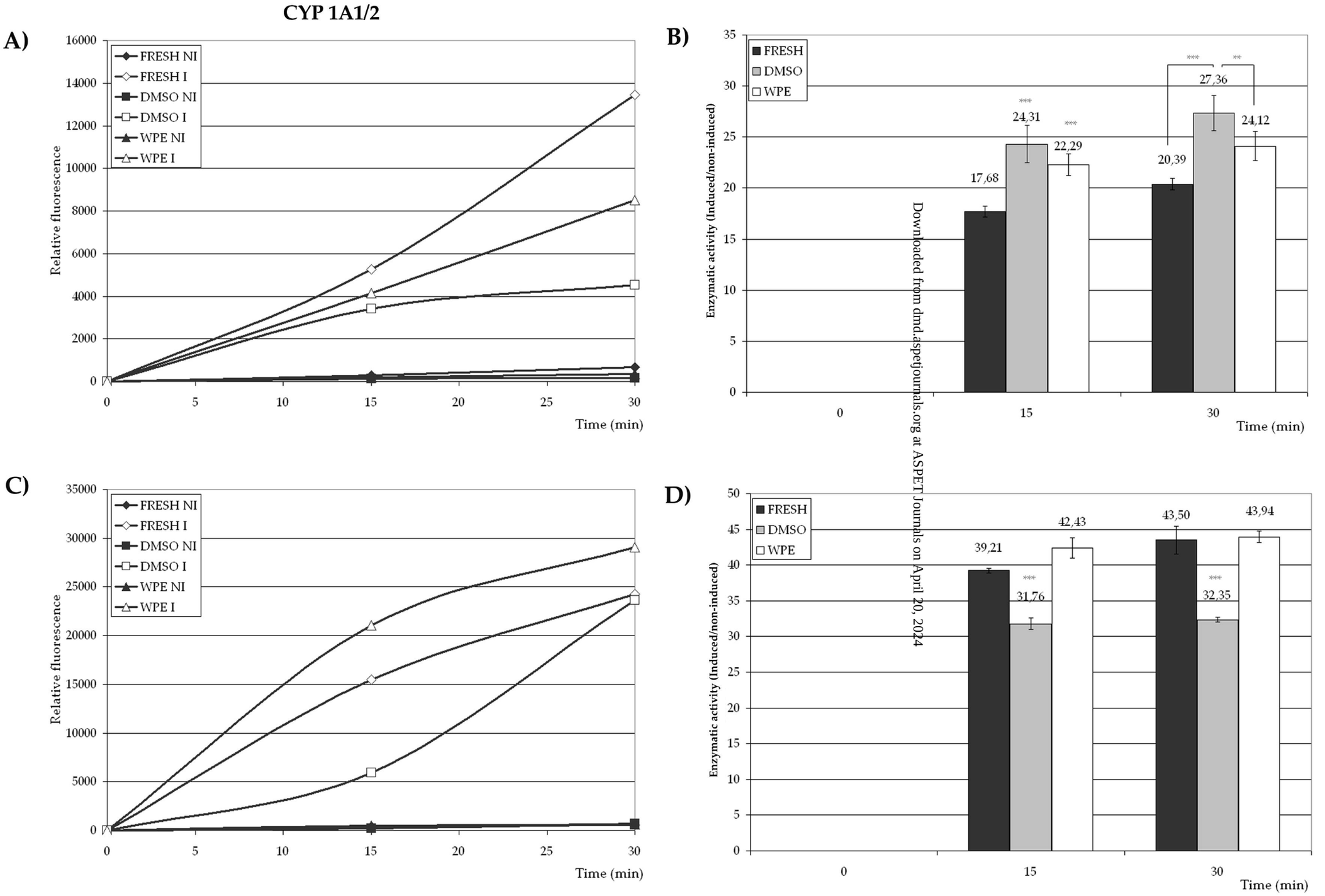

Figure 6